Available online at www.sciencedirect.com
ScienceDirect
Antiviral Research 75 (2007) 152–158
Antiviral Research
www.elsevier.com/locate/antiviral

Suppression of virus replication via down-modulation of mitochondrial short chain enoyl-CoA hydratase in human glioblastoma cells

Megumi Takahashi [a], Eiji Watari [a], Eiji Shinya [a], Takako Shimizu [b], Hidemi Takahashi [a,*]

[a] *Department of Microbiology and Immunology, Nippon Medical School, 1-1-5 Sendagi, Bunkyo-ku, Tokyo 113-8602, Japan*
[b] *Department of Environmental Medicine, Nippon Medical School, 1-1-5 Sendagi, Bunkyo-ku, Tokyo 113-8602, Japan*

Received 17 August 2006; received in revised form 17 February 2007; accepted 21 February 2007

Abstract

Several viruses have been demonstrated to be the etiologic agent in chronic progressive diseases, associated with persistence; however, major questions concerning the pathogenic mechanisms of viral persistence are still unanswered. With the aim of identifying host cellular proteins that may play a role in viral replication, we established long-term persistently infected human glioblastoma cell lines with mutant measles virus (MV) and analyzed the host proteins by two-dimensional gel electrophoresis (2-DE) with mass spectrometry. We observed significant down-modulation in the expression of mitochondrial short chain enoyl-CoA hydratase (ECHS), which catalyzes the β-oxidation pathway of fatty acid. Knockdown of this gene by a short interference RNA (siRNA) apparently impaired wild-type MV replication and the cytopathic effects (CPEs) of MV were significantly reduced in siRNA-transfected cells. These findings will shed light upon a new important notion for the interaction between virus replication and lipid metabolism in host cells and might provide a new strategy for virus control.

Keywords: Measles virus; Persistent infection; Mitochondrial short chain enoyl-CoA hydratase; β-Oxidation; Short interference RNA

1. Introduction

A persistent viral infection is one in which a virus in a replicating or non-replicating form persists in the host beyond the normal recovery and elimination period for that particular viral infection. Although the dynamics of immune responses after acute viral infection are well studied and very consistent, the patterns of responses noted during persistent infection are more complex and differ depending on the infection. Two essential ingredients have been identified in the current understanding of persistent virus infection. The first is an immune response that is ineffectual in recognizing and clearing a virus and/or virus-infected cells; the second is that viruses can regulate the expression of both their own genes and host genes to achieve residence in a non-lytic state within the cells they infect. However, knowledge of how viral genes and cellular factors interact to cause persistence is incomplete in most instances.

In our laboratory, we have established several monkey kidney cell lines persistently infected with temperature-sensitive mutants of measles virus (MV) (Watari et al., 1979, 2001). Many aspects of these cells such as interferon production have been investigated but fail to provide a coherent mechanistic explanation for viral persistence. In this study we established a human glioblastoma cell line persistently infected with mutant measles virus, because MV persistently infect and replicate in human cells of neuronal origin and elicit subacute sclerosing panencephalitis (SSPE) in humans (Horta-Barbosa et al., 1969; Payne et al., 1969). We analyzed these persistently infected cells using two-dimensional gel electrophoresis (2-DE) in combination with tandem mass spectrometry (MS/MS), which allows us to study the alterations of host proteins during virus adaptation to the cells.

Here, we found that the expression level of mitochondrial short chain enoyl-CoA hydratase (ECHS), which catalyzes the β-oxidation pathway of fatty acid, was specifically down-modulated in persistently infected cells. Moreover, knockdown of the gene by short interference RNA (siRNA) apparently impaired wild-type MV replication, and cytopathic effects (CPEs) by MV infection were significantly reduced in siRNA-transfected cells. If one of the hallmarks of persistent infection is stable and low-level virus replication, our findings suggest that some host cellular proteins associated with lipid metabolism

* Corresponding author. Tel.: +81 3 3822 2131x5381; fax: +81 3 3316 1904.
E-mail address: htkuhkai@nms.ac.jp (H. Takahashi).

0166-3542/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.antiviral.2007.02.002

might contribute to the regulation of virus replication followed by the establishment of persistent infection.

2. Materials and methods

2.1. Cell culture and viruses

Human glioblastoma cells, A172 and U373MG (Bender et al., 1992), were gifts from Dr. Hiroshi Takahashi (Department of Neurosurgery, Nippon Medical School, Tokyo, Japan) and were grown in Eagle's minimum essential medium (MEM, Nikken BioMedical Laboratory, Tokyo, Japan) supplemented with 10% heat-inactivated fetal calf serum, 100 U/ml of penicillin and 100 μg/ml of streptomycin. As described recently (Watari et al., 2005), wild-type Edmonston strain measles virus (Rapp clone 5) was grown and titrated on Vero cells. A temperature-sensitive mutant virus P-448 was established from Rapp clone 5 described previously (Yamaji et al., 1975). To establish persistently infected cells, A172 cells grown as monolayers were infected with P-448 mutant virus at a multiplicity of 0.1. Infected cells were passaged for the first time at 24 days' post-infection. Since the first passage, cells have been passaged weekly and were termed 448-A172 cells. For virus titration, serial tenfold dilutions of cell supernatants and cell lysates were inoculated into each of four wells of Vero cells and then incubated for 5 days. After incubation, wells were scored for CPE and we determined the dilution as $TCID_{50}$/ml at which 50% of the wells were infected.

2.2. Cell staining

For morphological analysis, cells grown on the culture plate were washed with PBS and fixed in 4% paraformaldehyde or acetone for 10 min, then cells were stained with hematoxylin solution. For immunochemical analysis, a cytospin preparation of A172 cells infected with wild-type MV and 448-A172 cells was incubated with vaccinated human serum with MV. After washing, they were overlaid with fluorescein isothiocyanate-conjugated goat anti-human antibody (Tago, Inc., Burlingame, CA).

2.3. Preparation of protein samples

The cell pellet (5×10^6 cells/sample) was disrupted in sample re-hydration buffer (8 M urea, 2% CHAPS, 0.5% ZOOM Carrier Ampholytes, 20 mM dithiothreitol (DTT), 0.002% bromophenol blue; Invitrogen, Carlsbad, CA) at room temperature for 15 min. The lysate was separated by centrifugation at $10{,}000 \times g$ for 5 min to yield supernatant that was stored at −80 °C until use. To visualize low-abundance proteins more efficiently, we prepared sub-cellular fraction of cells using a proteome extraction kit (Calbiochem, Darmstadt, Germany).

2.4. Two-dimensional gel electrophoresis

Cell lysate in re-hydration buffer was applied to ZOOM strips (pH 4–7, Invitrogen) in a total volume of 155 μl. After re-hydration for 16 h at room temperature, proteins were separated by isoelectrofocusing (IEF) at room temperature and 50 mA/strip with the following linear voltage increases: 200 V for 20 min, 450 V for 15 min, 750 V for 15 min, and 2000 V for 30 min. The strips were equilibrated in 50 mM Tris containing 6 M urea, 30% glycerol, 2% sodium dodecylsulfate (SDS) and 2% DTT for 20 min. The second dimension was performed on 13% SDS-polyacrylamide gels. Separated protein spots were fixed and stained on the gel with a silver staining kit (Nacalai Tesque, Kyoto, Japan). Differential spots were excised from silver-stained gels and treated with 20 μg of trypsin/ml in 50 mM ammonium bicarbonate buffer at 37 °C overnight. After in-gel digestion, the digested solution was transferred into a clean tube and dried under vacuum. The resulting samples were dissolved in 20 μl of 2% acetonitrile and 0.1% trifluoroacetic acid, and applied to LC–MS/MS analysis.

2.5. Identification of protein spots

Analysis was performed using an LC–MS/MS system with RP-mLC composed of a Paradigm MS4 dual solvent delivery system (Michrom BioResources, Auburn, CA), a HTC PAL auto sampler with two 10-port injector valves (CTC Analytics), Finnigan LCQ Deca XP plus (Thermo Electron, Waltham, MA) equipped with NSI sources (AMR Inc., Tokyo, Japan). The mass spectrometer was operated in data-dependent acquisition mode in which MS acquisition with a mass range of m/z 450–2000 was automatically switched to MS/MS acquisition under the automated control of Xcalibur software. The capillary exit of the electrospray ion source was set at 70 V, the octapole at 3 V, and the capillary temperature at 250 °C. A counter flow of helium was used as nebulizing gas. Each sample was injected onto a capillary RP column, MAGIC C18 (3 mm, 200 Å, 50 m × 0.2 m i.d., Michrom BioResources) with an acetonitrile linear gradient of 3 ml/min in formic acid 0.1%, from 2 to 60%. The HPLC column was rinsed with 90% acetonitrile in 0.1% formic acid between each injection.

2.6. siRNA transfection

A172 cells were plated in 48-well tissue culture plates at 1×10^5 cells/well in 150 μl MEM on the day of transfection. Cells were transfected with 1 μl HiPerFect Transfection Reagent (Qiagen, Düsseldorf, Germany) and 1 μl siRNAs (5 nM) in a total volume of 100 μl DMEM (Sigma–Aldrich, St. Louis, MO) according to the manufacturer's protocol. At 48 h after transfection, cells were infected with viruses and their growth on transfected cells was analyzed using the $TCID_{50}$ protocol. The siRNA oligonucleotides targeted the ECHS gene at position 864–884 (termed #864: aaagagaaaggccaacttcaa), 865–885 (termed #865: aagagaaaggccaacttcaaa), 1088–1108 (termed #1088: ctggqgcqccttctaaatcta), and 1245–1265 (termed #1245: cagatgctgattaaagtgata). These siRNAs were synthesized by Qiagen. Non-silencing siRNA with no known homology to mammalian genes was a commercially available duplex (Qiagen) and was used as control siRNA.

2.7. Quantitative RT-PCR

RNA was prepared from siRNA-transfected A172 cells using RNAeasy (Qiagen). One microgram of RNA was incubated for 1 h at 42 °C after adding 20 U of RNase inhibitors (TaKaRa, Bio Inc., Otsu, Japan), 0.2 mM deoxynucleoside triphosphates, 2.5 nM random primers, 11 U of Rous associated virus 2 reverse transcriptase (TaKaRa) and reverse transcriptase buffer to a final volume of 20 μl. One microlitre of RT reaction mixture was used as a template for real-time PCR using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) with the following primers specific for ECHS (forward, cgctgctgtcaatggctatg, and reverse, cttggcgtcctgggctgaga), β-actin (forward, tcacccacactgcccatctacga, and reverse, cagcggaaccgctcattgccaatgg), MV-NP protein (forward, tcagtagagcggttggaccc, and reverse, ggcccggtttctctgtagct), MV-H protein (forward, ttcatcgggcagccatctac, and reverse, ctctgaggtgtcctcaggcc), MV-F protein (forward, gcgagcctggaaactactaatca, and reverse, ccctgaacagccaatatcatctc). The amount of each ECHS mRNA was normalized to that of β-actin mRNA in the same sample.

2.8. Measurement of cell growth

Single cell suspensions were seeded at a density of 5×10^4 cells/well on 96-well microtiter plates. After 1–3 days of incubation, the cells were pulse-labeled with 0.5 μCi methyl-^{3}H-thymidine/well for the last 8 h, and were then harvested and counted using a β-counter (1450 Microbeta Trilux; Wallac, Gaithersburg, MD).

2.9. Western blotting

A172 cells treated with or without siRNA were infected with MV. After 2 days' infection, cells were lysed in 30 μl of lysis buffer (1% Nonidet P-40, 140 mM NaCl, 20 mM Tris–HCl (pH 8.0), 2 mM EDTA, 1 mM sodium orthovanadate, 1 mM PMSF, and 50 mM monoiodoacetamide) on ice for 15 min. After centrifugation at 20,400 × *g* for 15 min, proteins in cell lysates were separated by 10% SDS-PAGE under reducing conditions and transferred to a nylon membrane. The blots were probed with vaccinated human serum with MV or mouse anti-β-actin (clone AC-74, Sigma, St. Louis, MO) followed by peroxidase-

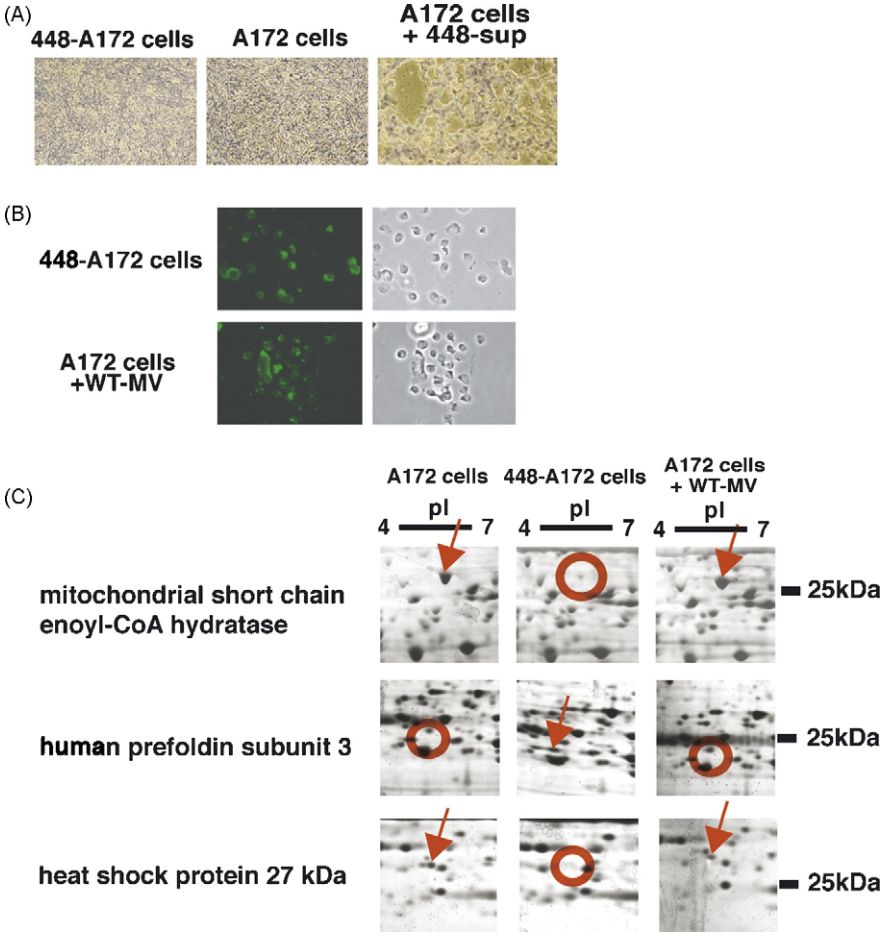

Fig. 1. Characterization of 448-A172 cells persistently infected mutant MV. (A) Morphology of 448-A172 cells (left), A172 cells (middle), and A172 cells treated with supernatants from 448-A172 cells. Cells were stained with hematoxylin solution. (B) Detection of virus antigens with vaccinated human serum with MV in 448-A172 cells (upper) or A172 cells infected with wild-type MV (lower). (C) Silver-stained two-dimensional gels of A172 cells (left column), 448-A172 cells (middle column), or A172 cells infected with wild-type MV (right column). Mitochondrial short-chain enoyl-CoA hydratase (ECHS) was detected in whole cell fraction, human prefoldin subunit 3 was detected in cytosolic fraction, and heat shock protein 27 kDa was detected in nucleus fraction.

conjugated rabbit anti-human IgG (MP Biomedicals, Irvine, CA) or goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA). Bands were visualized using a tetramethylbenzidine substrate kit (Vector, Burlingame, CA).

3. Results

3.1. Establishment and analysis of a cell line persistently infected with temperature-sensitive mutant measles virus

To investigate the mechanisms underlying virus persistence, we established a human glioblastoma cell line persistently infected with a temperature-sensitive mutant MV named 448-A172 after about 50 days of infection. The appearance of the 448-A172 cell line was indistinguishable from intact uninfected A172 cells (Fig. 1A). Thus, the situation of persistent MV infection in 448-A172 cells was examined by the detection of intracellular viral antigens using an immunofluorescent technique. As shown in Fig. 1B, viral antigens were mainly observed in the cytoplasms of 448-A172 cells, and infectious virions from cells could be obtained and titrated on Vero cell monolayers. Indeed, culture supernatants harvested on day 4 contained measurable amounts of viruses (10^4 $TCID_{50}$/ml) that induced syncytial cell formation for intact A172 cells (Fig. 1A).

3.2. Identification of proteins crucial for persistent infection in the established 448-A172 cell line

These findings suggest that such persistent infection did not depend on the type of virion but rather on host cellular conditions; therefore, to explore the possible mechanisms involved in persistent infection, we precisely compared the cellular proteins between 448-A172 cells and A172 cells infected with or without wild-type MV using 2-DE with p*I* values in the range of 4–7 to obtain a greater resolution in protein separation. The 2-DE image of cellular proteins after silver staining is shown in Fig. 1C. For the assessment of differentially expressed proteins, protein spots clearly altered in 448-A172 cells were considered. We did find three altered proteins, which were then characterized by mass spectrometry and identified as mitochondrial short chain enoyl-CoA hydratase, human prefoldin subunit 3, and heat shock protein 27 kDa.

3.3. Inhibition of ECHS expression with specific siRNA

Among those three proteins, we focused on investigating the functional role of ECHS for viral replication, because ECHS protein is more abundant than other proteins in intact A172 cells and the amount of ECHS is obviously reduced in 448-A172 cells. Using quantitative RT-PCR analysis quantifying ECHS transcripts relative to that of β-actin from 448-A172 cells, we confirmed that the expression levels of ECHS mRNA in 448-A172 cells was decreased to less than 10% in comparison with intact A172 cells (data not shown). Then, ECHS-specific siRNAs were prepared to evaluate the potential involvement of ECHS in regulating the replication of MV. Four different siRNAs were used to specifically knockdown the expression of ECHS in intact

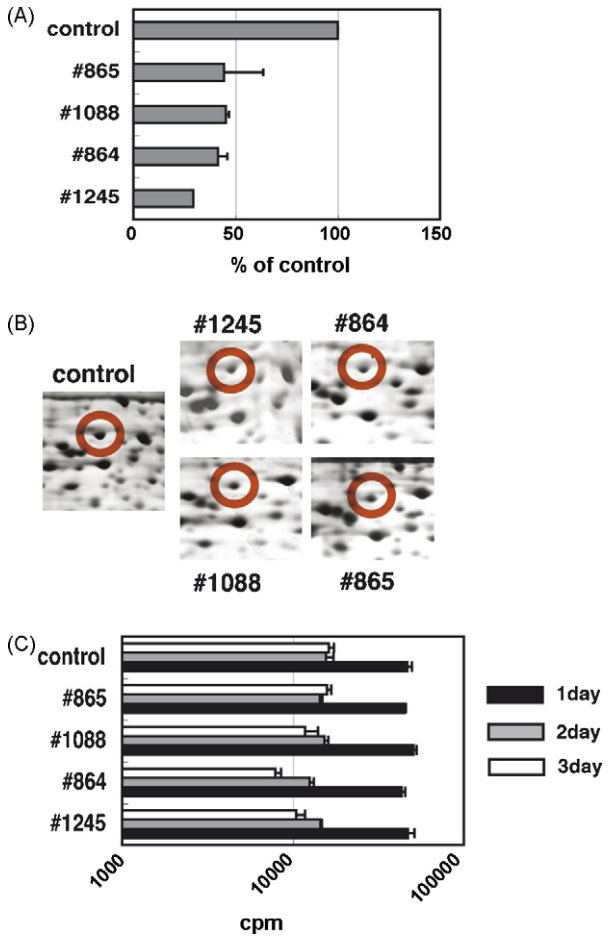

Fig. 2. Effect of siRNA transfection on A172 cells. (A) A172 cells were transfected with each siRNA. Total RNA was isolated from cells 24 h after transfection and was subjected to quantitative RT-PCR specific for ECHS or β-actin primer. Data were normalized to the amount of β-actin mRNA and are expressed as percentages of the normalized value for control siRNA-transfected cells. Values are the mean ± standard deviation (S.D.) of at least three experiments. (B) A172 cells were transfected with each siRNA. After 48 h incubation, cells were lysed and subjected to 2-DE analysis. Gels were visualized with silver staining. (C) A172 cells were transfected with each siRNA. After 1–3 days' incubation, the cells were pulse-labeled with 0.5 μCi/well methyl-^{3}H-thymidine for the last 8 h.

A172 cells. Twenty-four or 48 h post-transfection with a siRNA, mRNA and protein levels of ECHS in A172 cells were reduced up to 30–45% of control siRNA-transfected cells (Fig. 2A and B). As ECHS catalyzes the second step in the β-oxidation pathway of fatty acid metabolism, down-modulation of ECHS might result in the deficient production of energy-yielding substrates via β-oxidation. Therefore, we examined the effect of ECHS suppression on cell proliferation using the ^{3}H-thymidine uptake method. Although siRNA had little effect on cell proliferation until 2 days after transfection, the reduction of ^{3}H-thymidine uptake was observed in three siRNA-transfected cells, but no dead cells were detected 3 days after transfection (Fig. 2C).

3.4. Inhibition of MV replication by ECHS siRNA in A172 cells

To see whether the treatment of cells with ECHS-specific siRNA can inhibit MV replication, we examined their inhibitory

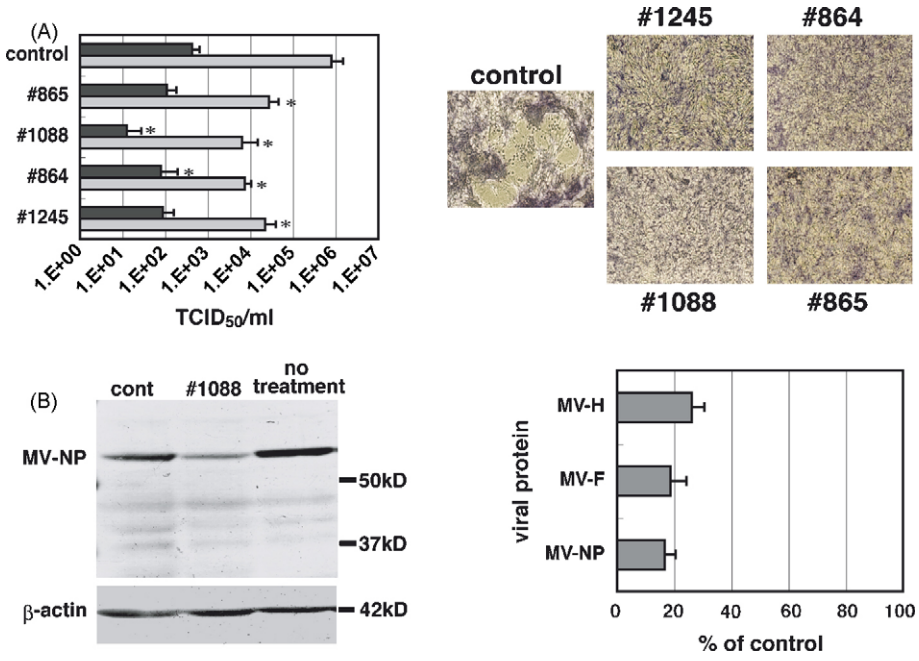

Fig. 3. Effect of siRNA transfection on MV replication. (A) A172 cells were transfected with each siRNA. After 48 h incubation, A172 cells were infected with wild-type MV, and then both cells (gray bars) and supernatants (charcoal bars) were harvested at 48 h after infection followed by determination of the virus titer (left). Data are expressed as the virus titer ($TCID_{50}$) and are the means ± S.D. of values of at least three independent experiments. $^{*}P < 0.05$ vs. the value of cells transfected with control siRNA (Student's *t*-test). Cells were stained with hematoxylin solution after 48 h infection (right). (B, left) A172 cells treated with or without siRNA were infected with MV. After 2 days' infection, cells were lysed in 30 μl of lysis buffer, and proteins in lysates were separated in 10% polyacrylaminde gel and blotted on a nylon membrane. The blot was probed with vaccinated human serum with MV (upper column) or mouse anti-β-actin antibody (lower column). (B, right) A172 cells treated with or without siRNA were infected with MV. After 2 days' infection, total RNA was isolated from cells 24 h after transfection and subjected to quantitative RT-PCR specific for MV-NP protein, MV-F protein or MV-H protein primer. Data were normalized to the amount of β-actin mRNA and are expressed as percentages of the normalized value for control siRNA-transfected cells. Values are the mean ± S.D. of at least three experiments.

effect on intact A172 cells. Forty-eight hours post-transfection with siRNA, A172 cells were infected with wild-type MV at a multiplicity of infection (moi) of 0.1. At 48 h after infection, culture supernatants and cells were harvested, serially diluted, and the virus titer determined (expressed as $TCID_{50}$/ml). As shown in Fig. 3A, we observed the efficient inhibition of MV replication after transfection with four distinct siRNAs. Indeed, when #1088 siRNA was used, inhibition was so pronounced that culture supernatants contained only a few viruses. CPE by MV infection observed at 48 h post-infection in control and ECHS siRNA-transfected groups is also shown in Fig. 3A. There is an apparent marked reduction of CPE in all siRNA-transfected cells. This was confirmed by titrating the virions yielded between control and siRNA-transfected cells. Moreover, Western blot analysis showed that transfection of A172 cells with #1088 siRNA reduced the accumulation of viral protein compared to cells transfected with control siRNA or without siRNA. This protein reduction was due to a decrease in the expression of viral mRNA (Fig. 3B).

3.5. *Effect of ECHS siRNA on other virus replication*

Next, to determine if ECHS is also involved in the replication of other RNA viruses, siRNA-transfected A172 cells were infected with vesicular stomatitis virus (VSV) or semliki forest virus (SFV). As demonstrated in Fig. 4A, targeting ECHS mRNA also significantly inhibited both VSV and SFV replication in A172 cells. Similar to MV infection, CPEs were not detected in all siRNA-transfected A172 cells at 24 h after VSV or SFV infection (data not shown).

ECHS catalyzes the β-oxidation pathway of fatty acid. To further analyze the involvement of β-oxidation in virus replication, we examined the effect of etomoxir (Sigma), an inhibitor of carnitine palmitoyltransferase that inhibits mitochondrial β-oxidation, on MV replication in A172 cells. Treatment of A172 cells with etomoxir resulted in the suppression of MV replication in a dose-dependent manner (Fig. 4B). These results suggest that β-oxidation might be involved in MV replication.

We also observed that treatment of A172 cells with IFN-β (1000 IU/ml) effectively inhibited MV replication by approximately 100 times (Fig. 4B), indicating that down-modulation of ECHS potency with siRNA corresponds to treatment with a high titer of IFN-β to inhibit virus replication. Recently, it was reported that siRNA treatment could nonspecifically induce IFN-mediated innate immune responses (Sledz and Williams, 2004); however, it was unlikely that IFN mediated protection in our experiments, because a large amount of IFN-α was originally produced in intact A172 cells but IFN-β was not detected in culture supernatants from intact or siRNA-transfected A172 cells (data not shown).

To extend the results in A172 cells, we further tested the ability of siRNAs to inhibit virus replication in other glioblastoma cells, U373MG. When U373MG cells were transfected with each siRNA and then infected with MV at a moi of 0.1

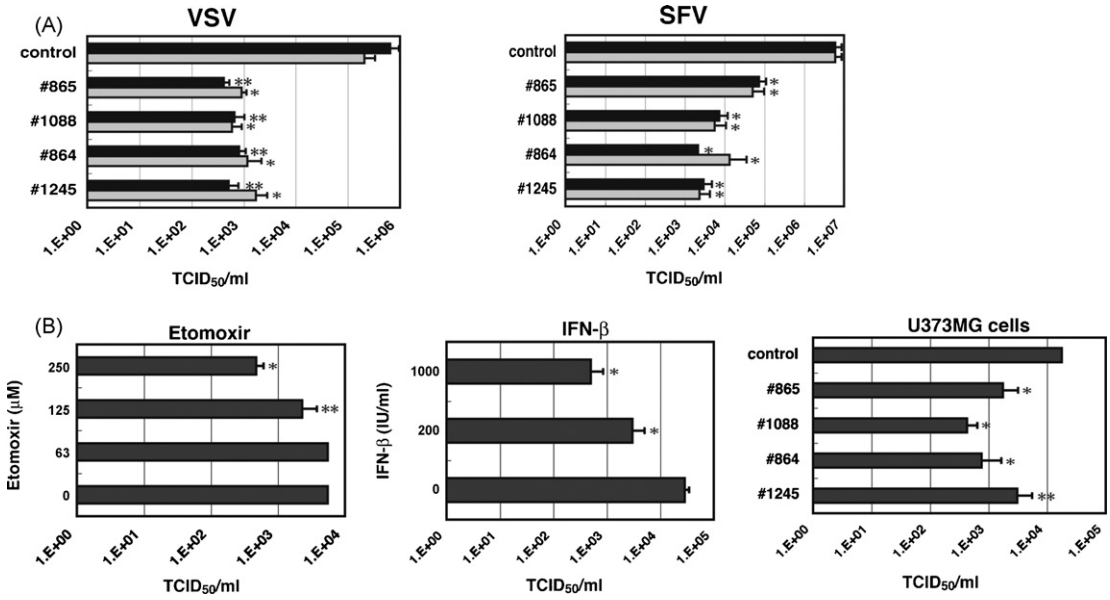

Fig. 4. Effect of siRNA transfection on other virus replication. (A) A172 cells were transfected with each siRNA. After 48 h incubation, A172 cells were infected with VSV (left) or SFV (right) at a moi of 0.1, and then cells (gray bars) and supernatants (charcoal bars) were harvested at 24 h after infection followed by determination of the virus titer. (B) A172 cells were treated with the indicated concentration of etomoxir (left). After 48 h incubation, A172 cells were infected with wild-type MV, and then supernatants were harvested at 72 h after infection followed by the assay for determination of the virus titer. A172 cells were treated with the indicated concentration of IFN-β (middle). After 24 h incubation, cells were infected with wild-type MV, and then supernatants were harvested at 48 h after infection followed by determination of the virus titer. U373MG cells were transfected with each siRNA (right). After 48 h incubation, U373MG cells were infected with wild-type MV, and then supernatants were harvested at 48 h after infection followed by determination of the virus titer. Data are expressed as the virus titer ($TCID_{50}$) and are the means ± standard deviations (S.D.) of values of at least three independent experiments. (A and B) $^{*}P < 0.02$, and $^{**}P < 0.05$ vs. the value of cells transfected with control siRNA or treated without reagents.

at 48 h post-transfection, siRNA, especially #864 and #1088, significantly inhibited virus replication (Fig. 4B). Moreover, to confirm whether stable infection with mutant MV in other cells induce the down-modulation of ECHS expression as well as 448-A172 cells, we are now attempting to establish a cell line persistently infected with mutant MV using U373MG cells.

4. Discussion

In this study, we have found that the expression of ECHS protein was significantly down-modulated in cells persistently infected with temperature-sensitive mutant MV. Moreover, similar to conventional MV, the mutant MV produced and secreted from persistently infected cells showed the capacity to induce syncytial formation upon infection to normal A172 human glioblastoma cells, indicating that such persistent infection is not due to the type of virion but rather to host cellular conditions. We thus speculate the ECHS gene as an essential host cellular gene utilized for virus replication, and conducted experiments to inhibit host cellular ECHS with siRNA by the gene-transfer technique. As expected, the replication of wild-type MV was specifically blocked by siRNA via knockdown of the ECHS gene.

ECHS catalyzes the second step in the physiologically important β-oxidation pathway of fatty acid metabolism (Agnihotri and Liu, 2003). In mammals, fatty acid oxidation occurs in mitochondria, peroxisomes, and smooth endoplasmic reticulum. Although mitochondria and peroxisomes oxidize fatty acids via β-oxidation, smooth endoplasmic reticulum metabolizes fatty acids by ω-oxidation. Such mitochondrial β-oxidation is responsible for oxidation in the major portion of short- (<C8), medium- (C8-12), and long- (C14-20) chain fatty acids and, in that process, constitutes the primary source of energy derived from fatty acids. The catalytic mechanism of ECHS has been studied in great depth through a combination of kinetic, spectroscopic, and structural techniques (Kim and Battaile, 2002); however, the expression and gene regulation of this enzyme have not been fully elucidated. Several reports have shown that the expression level of ECHS was decreased in cancer cell lines and carcinoma (Balabanov et al., 2001; Fratelli et al., 2003; Hwa et al., 2005; Sakata et al., 1998). Although the biological significance of ECHS in human cancer has not been confirmed, regulation of this enzyme by a carcinogen might have a role in the proliferation and differentiation of normal cells. On the other hand, very few reports refer to the interaction between ECHS and microbial infections. Only Yokoyama et al. have reported that the expression level of ECHS was decreased in hepatocellular carcinoma from patients infected with hepatitis C virus (Yokoyama et al., 2004).

Although we have not yet elucidated the molecular mechanism for the regulation of MV replication by ECHS, down-modulation of ECHS inevitably leads to the inhibition of β-oxidation. We also found that treatment of A172 cells with etomoxir, an inhibitor of β-oxidation, effectively suppressed virus replication. These findings suggest that β-oxidation appeared to be involved in MV replication; moreover, the fact that ECHS

siRNA also effectively interfered with VSV or SFV replication in A172 cells indicates that β-oxidation might be essential for the common replication cycles of various viruses.

Recently, there have been significant advances in identifying cellular factors that promote or inhibit viral replication. In the case of HIV-1, novel factors such as APOBEC or TRIM5α have been discovered (Sheehy et al., 2002; Stremlau et al., 2004). Both proteins show anti-viral activity; in particular, APOBEC family proteins have activity in a wide variety of viruses. Consequently, up-modulation of these proteins in host cells imparts resistance to viral replication. Taken together, there may be two concepts for suppressing virus replication in host cells: the lack of an essential factor for virus replication and the presence of an efficient mechanism for controlling replication. APOBEC proteins seem to correspond only to the latter mechanism, while ECHS might correspond to both, because the impairment of β-oxidation might result in the reduction of energy-yielding substrates (acetyl-CoA and ketone bodies), which eventually leads to a shortage of ATP, and adversely cause the accumulation of both free fatty acids and toxic acyl-CoA intermediates in cells.

Although the expression level of ECHS in 448-A172 cells seemed significantly lower than that in siRNA-transfected cells, culture supernatants from the 448-A172 cells still contained measurable amounts of viruses (10^4 $TCID_{50}$/ml) and their growth rate was almost the same as #864-siRNA-transfected cells. These data suggest that other host factors relating to viral replication besides ECHS might be stably disturbed in 448-A712 cells. Indeed, we found that human prefoldin subunit 3 and heat shock protein 27 kDa seemed to down-modulate virus replication and extend cell survival (Fig. 1C). Further investigation on another host proteins must be required to understand the precise mechanisms how viral genes and cellular factors interact to cause persistent viral infection.

One of the hallmarks of persistent infection is to create an excellent intracellular status for stable and low-level virus replication. The reduction of ECHS may contribute not only to low steady-state levels of virus replication but also to the survival of virus-infected cells. The suppression of virus replication might help to prolong the asymptomatic phase of virus infection; therefore, further precise analysis of the molecular regulation of our findings shown here might cast new light on the development of novel anti-viral drugs concerned with lipid metabolism. Indeed, Sakamoto et al. recently identified an HCV replication inhibitor which prevents the de novo synthesis of sphingolipids, a major lipid raft component (Sakamoto et al., 2005).

Acknowledgements

We thank Drs. Takahiro Isono, Noriyuki Nagahara and Masayasu Minami for experimental advice on the proteomic analysis. This work was supported in part by grants from the Ministry of Education, Science, Sport, and Culture, from the Ministry of Health and Labor and Welfare, Japan, from the Japanese Health Sciences Foundation, and from the Promotion and Mutual Aid Corporation for Private School of Japan.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.antiviral.2007.02.002.

References

Agnihotri, G., Liu, H.W., 2003. Enoyl-CoA hydratase. reaction, mechanism, and inhibition. Bioorg. Med. Chem. 11, 9–20.

Balabanov, S., Zimmermann, U., Protzel, C., Scharf, C., Klebingat, K.J., Walther, R., 2001. Tumour-related enzyme alterations in the clear cell type of human renal cell carcinoma identified by two-dimensional gel electrophoresis. Eur. J. Biochem. 268, 5977–5980.

Bender, H., Takahashi, H., Adachi, K., Belser, P., Liang, S.H., Prewett, M., Schrappe, M., Sutter, A., Rodeck, U., Herlyn, D., 1992. Immunotherapy of human glioma xenografts with unlabeled, 131I-, or 125I-labeled monoclonal antibody 425 to epidermal growth factor receptor. Cancer Res. 52, 121–126.

Fratelli, M., Demol, H., Puype, M., Casagrande, S., Villa, P., Eberini, I., Vandekerckhove, J., Gianazza, E., Ghezzi, P., 2003. Identification of proteins undergoing glutathionylation in oxidatively stressed hepatocytes and hepatoma cells. Proteomics 3, 1154–1161.

Horta-Barbosa, L., Fuccillo, D.A., London, W.T., Jabbour, J.T., Zeman, W., Sever, J.L., 1969. Isolation of measles virus from brain cell cultures of two patients with subacute sclerosing panencephalitis. Proc. Soc. Exp. Biol. Med. 132, 272–277.

Hwa, J.S., Park, H.J., Jung, J.H., Kam, S.C., Park, H.C., Kim, C.W., Kang, K.R., Hyun, J.S., Chung, K.H., 2005. Identification of proteins differentially expressed in the conventional renal cell carcinoma by proteomic analysis. J. Kor. Med. Sci. 20, 450–455.

Kim, J.J., Battaile, K.P., 2002. Burning fat: the structural basis of fatty acid beta-oxidation. Curr. Opin. Struct. Biol. 12, 721–728.

Payne, F.E., Baublis, J.V., Itabashi, H.H., 1969. Isolation of measles virus from cell cultures of brain from a patient with subacute sclerosing panencephalitis. N. Engl. J. Med. 281, 585–589.

Sakamoto, H., Okamoto, K., Aoki, M., Kato, H., Katsume, A., Ohta, A., Tsukuda, T., Shimma, N., Aoki, Y., Arisawa, M., Kohara, M., Sudoh, M., 2005. Host sphingolipid biosynthesis as a target for hepatitis C virus therapy. Nat. Chem. Biol. 1, 333–337.

Sakata, M., Kurachi, H., Morishige, K., Ogura, K., Yamaguchi, M., Nishio, Y., Ikegami, H., Miyake, A., Murata, Y., 1998. Messenger RNA differential display reverse-transcriptase-polymerase-chain-reaction analysis of a progestogen-suppressive gene in a human endometrial-cancer cell line. Int. J. Cancer 78, 125–129.

Sheehy, A.M., Gaddis, N.C., Choi, J.D., Malim, M.H., 2002. Isolation of a human gene that inhibits HIV-1 infection and is suppressed by the viral Vif protein. Nature 418, 646–650.

Sledz, C.A., Williams, B.R., 2004. RNA interference and double-stranded-RNA-activated pathways. Biochem. Soc. Trans. 32, 952–956.

Stremlau, M., Owens, C.M., Perron, M.J., Kiessling, M., Autissier, P., Sodroski, J., 2004. The cytoplasmic body component TRIM5alpha restricts HIV-1 infection in Old World monkeys. Nature 427, 848–853.

Watari, E., Yamanaka, A.M., Yamaji Y, M., 1979. Establishment of persistent infection of measles virus in Vero cells with special reference to the temperature-sensitivity of parent viruses. J. Nippon Med. Sch. 46, 147–150.

Watari, E., Shimizu, M., Takahashi, H., 2005. Langerhans cells stimulated by mechanical stress are susceptible to measls virus. Intervirology 48, 145–152.

Watari, E., Shinya, E., Kurane, S., Takahashi, H., 2001. Effects of cyclosporin A on cell fusion in a monkey kidney cell line persistently infected with measles virus. Intervirology 44, 209–214.

Yamaji, Y., Honda, B.F., Todome, H., Suganuma, Y., Watari, M., Iwaguchi, E., Nagashima M, H., 1975. Characterzation of temperature-sensitive mutants of measles virus: temperature-shift experiment. Jpn. J. Med. Sci. Biol. 28, 223–229.

Yokoyama, Y., Kuramitsu, Y., Takashima, M., Iizuka, N., Toda, T., Terai, S., Sakaida, I., Oka, M., Nakamura, K., Okita, K., 2004. Proteomic profiling of proteins decreased in hepatocellular carcinoma from patients infected with hepatitis C virus. Proteomics 4, 2111–2116.